Temporal Changes in Gene Transcription of *Salmonella* Typhimurium Induced by Pulsed Electric Field.

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Abstract

Pulsed electric field (PEF) is a novel food processing technology that promises to deliver safe foods with increased quality by inactivating potentially harmful microorganisms while generating a minimum thermal effect. The engineering aspects of this technology have been thoroughly studied and as a result, PEF is now feasible for commercial application. However, the mechanism of microbial inactivation via PEF is still unclear and must be resolved before the technology can be approved for commercial use. Theories of the mechanism of microbial inactivation of PEF via membrane disruption have been suggested but specific cellular targets have yet to be elucidated. The purpose of this study is to examine temporal changes in the transcriptome of PEF-treated *Salmonella* Typhimurium LT2 in order to elucidate the molecular basis for cellular inactivation. This knowledge will allow for more directed efforts to increase the killing efficiency of PEF and aid in the development standardized processing procedures for use in the food industry.

Briefly, *Salmonella* Typhimurium LT2 ATCC 19585 was inoculated into 5% strength tryptose soy broth (TSB), with electrical conductivity of 1.2 mS/m, and incubated at 32°C for 18-22 h with shaking at 150 rpm. This was followed by two additional passages of the inoculum under identical conditions. The final culture was collected at mid-log phase (7.3–7.5 log cfu/mL) for treatment. Cells of the final culture

were treated with a PEF processor (OSU-4ERRC) using a predetermined treatment that produced a 1-log cfu/mL reduction in cell population as determined by the plate count method on tryptose soy agar (TSA). The treatment conditions were: square wave, bipolar pulse, 25.6 kV/cm electric field, 3 μs pulse width, 1 ms delay time, and 3 mL/s flow rate, giving a total treatment time of 91 μs. The temperature of the treated culture was maintained at 30-32°C with cooling before the first chamber, after the second chamber, and after the fourth chamber, via cooling coils submerged in a circulating water bath. RNA of treated cells was extracted at 0 (control), 3, 30, and 60 min post treatment and used to produce labeled complementary DNA (cDNA) via Reverse Transcriptase PCR (RT-PCR). cDNA from opposing samples was labeled for comparison with either or Cy3 and Cy5 and opposing samples were competitively hybridized to a *Salmonella* microarray slide (Pathogen Functional Genomic Research Center *Salmonella* Typhimurium, Version 5) containing four replicates of each *Salmonella* open reading frame. The slides were manually washed and scanned at 10 μm spot resolution. Image and data analysis were performed using the TIGR TM4 software suite; specifically, Spotfinder v. 3.2.1 for image analysis, MIDAS v. 2.21 for data normalization, and Multiple Experiment Viewer (MeV) v 4.5.1 for statistical analysis and gene clustering. Data processing included spots identification using the Otsu algorithm, LOWESS normalization of spot intensities, standard deviation regularization, flip dye cross-slide replicate analysis, and in-slide replicate analysis. ANOVA between time points was performed using Pearson correlation distance metric with $p=0.02$ and fold change of 1.5 was used as the arbitrary cutoff ratio. All slides were duplicated and fold change of all samples was determined with respect to the 0 min. sample.

A total of 560 genes, representing ~2% of the genes in the *Salmonella* genome showed a significant difference in transcription between samples, and of these 276, representing ~1% of the genome had a fold change of ≥ 1.5. Genes related the DNA damage induced SOS response were prevalent among those unregulated at 30 and 60 min post treatment. These included genes of the *rec, umu, din, uvr,* and *rtc* operons, which are responsible for DNA damage repair and mutagenesis. Genes in the *wca*, *yjb, and psp* operons, which are associated with stress to the cell envelope and are responsible for exopolysaccharide production, showed a transient increase in transcription at 30 min post treatment. Flagellar synthesis genes in the *fli* and *flg* operons showed a transient decrease in transcription at 30 min post treatment.

Patterns of transient gene expression, which include genes mainly involved in maintenance of components of the cell envelope, suggest damage to cell membranes. This agrees with previous reports of the cellular inactivation mechanism attributed to PEF, especially the temporary nature of the damage as implied by the transient up regulation of response and repair genes. Sustained increase in the transcription of SOS genes suggests that cells also experience significant DNA damage as a result of PEF processing. The up regulation of SOS genes may also explain the reports of a recovery period post PEF processing during which damaged cells are viable, but non-recoverable. It is unclear as to whether DNA damage is a direct result of the applied electric field, or if DNA damage occurs indirectly, due to influx of damaging materials into the cell during the time that membrane integrity is reduced. The function of many genes remains to be investigated and most are expected to be related to the DNA and envelope damage repair pathways. However, due to the large number of genes yet to be investigated in this study,

it is likely that additional cellular targets of PEF will also be identified. As the full scope of cellular components affected by PEF is realized, methods for increasing the treatment's effectiveness against undesirable bacteria in foods will be more readily determined.

Introduction

Pulsed electric field (PEF) processing is a promising technology for the non-thermal treatment of select food products. Liquid foods such as eggs, juice, broth, and milk, as well as liquid foods containing small particulates, such as soups, are the primary candidates for PEF processing. However, powders that exhibit fluid-like flow can also be treated with PEF. When compared to traditional heat pasteurization, non-thermal processing causes less degradation of colors, flavors, vitamins, and potentially desirable enzymes. As the demand for minimally processed foods with fresh-like qualities increases, PEF processing has the potential to deliver the value added products desired by industry and the high quality products desired by consumers.

The advantages of PEF processing make it a desirable technology for food processors, but before this technology can be implemented, regulations and guidelines for safe and effective use must be established. Research towards these ends has lead to the development of continuous flow processors that produce consistent results and has identified the optimum range for the electric field strength, the most effective waveforms, and the most efficient pulse polarities for the killing of microorganisms. The death kinetics of an array of microorganisms under various conditions has been examined with

the goal of providing insights into methods of further improving the efficiency of PEF via technological adjustments or combinational treatments that provide a hurdle effect.

Current knowledge of the mechanism of microbial inactivation by PEF processing is based on observations of changes in membrane potential and permeability after PEF treatment. These studies were useful in the determination that PEF physically damages cells membranes, but they do not identify the specific sites of this damage or address other cellular components. Reports of injured cells after PEF treatment are inconclusive, suggesting the need for a more detailed explanation of the recovery of cells exposed to PEF. The purpose of this project is to investigate the transcriptional profile of *Salmonella* Typhimurium during recovery from sub-lethal PEF treatment. Detailed examination of the transcriptome will help to elucidate the specific repair and recovery systems activated in PEF-injured cells. Studying these cellular repair systems should reveal specific targets, which will aid in the selection and development hurdle treatments to combine with PEF processing. This knowledge may also aid in the development of media for the accurate enumeration of viable-but-injured cells present in foods after PEF treatment.

Materials and Methods

Culture preparation and PEF inactivation kinetics

Salmonella Typhimurium LT2 was prepared from frozen stock and incubated overnight at 32ºC in 5% strength tryptose soy broth (TSB) with shaking at 120 rpm. Cells were passed twice. Fresh media was then inoculated with 0.1% overnight culture and grown to mid-log phase (7.3-7.5 cfu/mL, conductivity: 1.0±0.1mS/cm). The

resulting culture was immediately treated in a PEF processor at 25.6 kV/cm, 32ºC±2ºC, and treatment time of 90.4 us. The control culture was passed though the PEF processor with no electric field applied (Fig. 1). Aliquots of control and treated cultures were collected immediately after treatment (~3 min) and plated to tryptose soy agar (TSA) for enumeration. The remaining volume of each culture was incubated at 32ºC and aliquots were plated at select time points (Fig. 2).

RNA extraction and cDNA synthesis

Cells were PEF-treated as described previously and RNA was extracted from cells collected at 3, 30, and 60 min post treatment. RNA was extracted from the control culture at 3 min. (Fig 1) . {Joe: According to Fig 1 title, controls for 30 and 60 min were also prepared}. RNA was treated with DNase I to remove contaminating DNA; subsequently, RNA integrity and concentration were measured via capillary gel electrophoresis and spectrophotometric measurement at A260, respectively. A reverse transcriptase reaction including aminoallyl-deoxyuraciltriphosphate (aa-dUTP) was used to produce complementary DNA (cDNA) with incorporated aminoallyl linkers. cDNA was labeled with either cy3 or cy5 fluorescent dye at aminoallyl sites and cy3, cy5, and cDNA concentration were measure for each sample and dye incorporation ratio was calculated using the following equations:

Cy3 incorporation: $\text{pmol Cy3} = \frac{OD_{550} * \text{volume (\_L)}}{0.15}$

Cy5 incorporation: $\text{pmol Cy5} = \frac{OD_{650} * \text{volume (\_L)}}{0.25}$

Dye incorporation ratio: # nucleotides/dye incorporated= $\frac{\text{pmol cDNA}}{}$

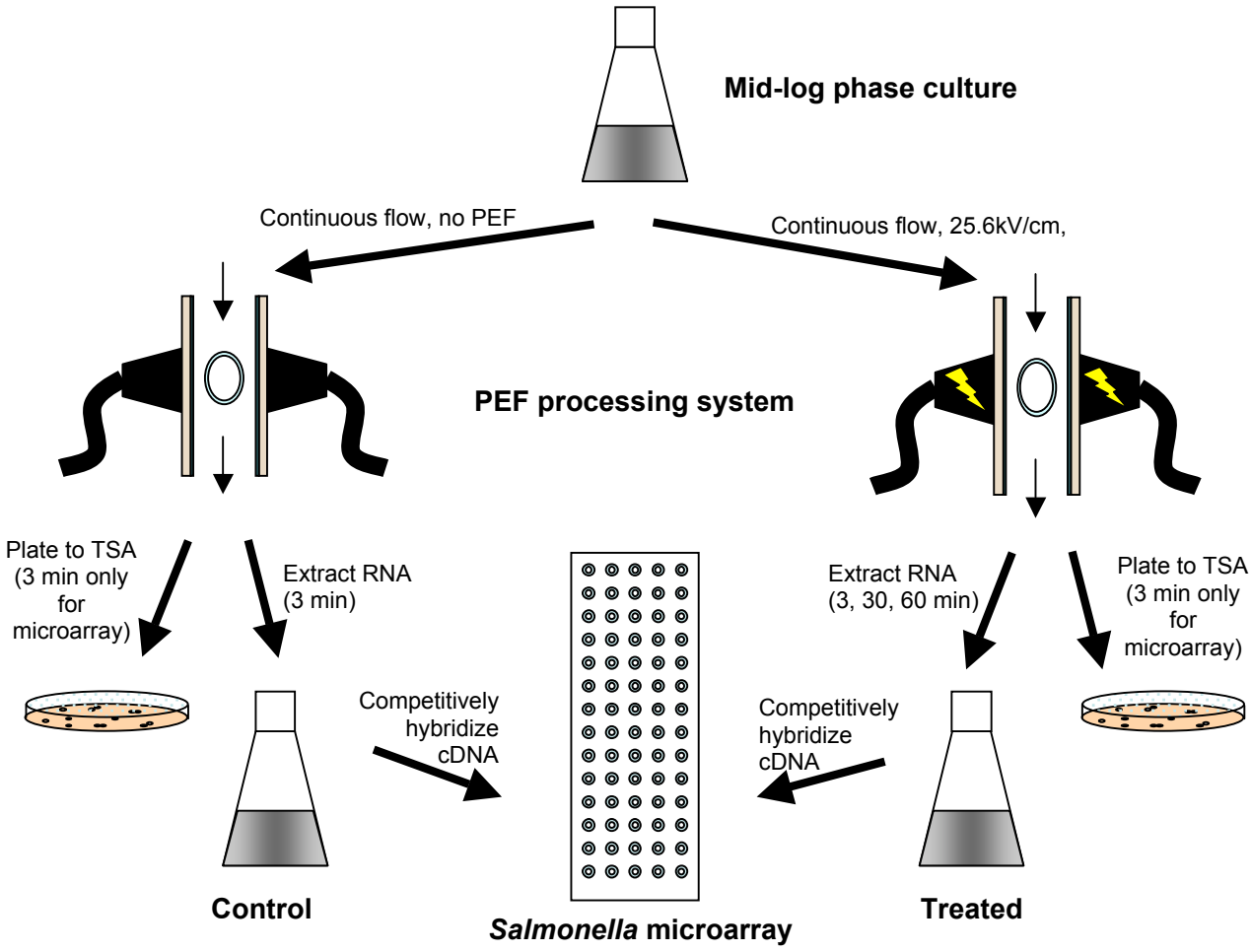

Figure 1. Simplified experimental design. Mid-log phase culture was divided with one portion being treated with pulsed electric field (PEF) at 25.6 kV/cm, 91 _s and 32ºC±2ºC, and the remaining portion passed through the PEF processor without applied electric field. Immediately after treatment (3 min) aliquots were plated to TSA for enumeration and RNA was extracted. The remaining treated culture was incubated at 32ºC and aliquots were taken for enumeration and RNA extraction at 30 min and 60 min after treatment.

pmol Cy dye

Microarray hybridization and scanning

Salmonella two-color microarray slides were prepared for hybridization by blocking with slide hybridization buffer, rinsing with MilliQ water, and drying with isopropyl alcohol followed by centrifugation for 2 min in a slide centrifuge. {Was the slide itself centrifuged? Yes.}. Meanwhile, oppositely labeled cDNA from treated and

control cultures was combined and dried in a vacuum centrifuge. The combined cDNA was resuspended in cDNA hybridization buffer and denatured at 95ºC for 10 min. A Maui Mixer slide cover was affixed to the microarray slide and ~50_L of cDNA solution was added. Mixers were sealed and slides were hybridized in a Maui hybridization station for 20h at 42ºC.. After hybridization, slides were washed in sequence with low, medium, and high stringency wash buffers followed by MilliQ water. Slides were dried in a slide centrifuge for 8 min and then scanned using excitation wavelengths of 532nm and 636nm for Cy3 and Cy5, respectively. Each slide {Do you scan the top and bottom of the same slide?}was scanned twice, first using manually determined gain values and saturation limits and the second time using gain values and saturation limits determined by GenePix Pro imaging software

Image analysis, Data normalization, Statistical analysis, and Clustering

Image and data analysis were performed using the TM4 software suite. Briefly, image analysis was performed using Spotfinder v. 3.2.1 with the Otsu algorithm for spot identification. Images were then examined visually for abnormalities, proper grid alignment, and accurate assignment of flags. Flagged spots were retained and included in data normalization. Data normalization was performed in MIDAS v. 2.21. Individual data sets were normalized using the LOWESS algorithm followed by standard deviation regularization the data set. Next flip dye analysis {Is that s technique or just description of the analysis? It is a technique} of oppositely labeled, duplicate slides was performed, resulting in a merged data set representing the average Cy3:Cy5. Finally, replicate analysis of replicate spots within the same slide was performed, producing overall Cy3:Cy5 for each gene. Statistical analysis was performed in Multiple Experiment

Viewer (MeV) v. 4.5.1. ANOVA was used to identify genes showing significant ($p=0.02$) changes in transcription over time. Significant genes were then clustered using the Self-Organizing Map (SOM) algorithm with Pearson Correlation as the distance metric in order to separate genes based on transcriptional profile. The resulting 6 clusters were then individually re-clustered using the SOM algorithm and Euclidean Distance as the distance metric in order to separate genes with similar transcriptional profiles based on $\log_2$ ratio. Data sets were transferred to Microsoft Excel software and genes were grouped by operon. Finally, a fold change of ≥ 1.5 was arbitrarily set as the cut-off for meaningful change.

Results and Discussion

PEF inactivation kinetics

After PEF treatment, *Salmonella* Typhimurium LT2 showed static cell count for at least two hours (Fig. 2). The average doubling time of the untreated culture from 4-6 h was 30 min. Assuming that the entire surviving population was undamaged, the population of the treated culture would be expected increase from 6.3 to 7.5 log cfu/mL during the two hours post treatment. It may not be reasonable to assume that the entire surviving population was undamaged. The methods used in this experiment may be reasonably expected to detect a population increase from 6.3 to 6.5 log cfu/mL, representing the doubling of 5.25 log cfu/mL, or 10% of the surviving population, at a rate of 30 min/generation over the course of two hours {I don't understand this statement!}. The observation that cells showed no growth suggests that at least 90% of

the surviving cells experienced sub-lethal injury and were in a recovery phase after treatment.

The Rcs regulon and EPS

Microarray analysis showed a transient increase in the transcription of several genes (Table 1) under the control of the Rcs regulon, which is capable of sensing and responding to membrane damage.{We need to include a brief description when a gene or operon is first introduced} (Ferrieres 2003). Genes and operons controlled under the Rcs regulon are activated by either RcsB or a combination of RcsA and RcsB (Majdalani 2005). RcsB is activated via phosphorylation by RcsD, a membrane bound histidine kinase and member of a two component regulatory system with inner membrane RcsC (Fig. 3). RcsC responds to various treatments that result in the alteration of the cell envelope, including desiccation, and osmotic shock, however the exact nature of the inducing signal has not yet been elucidated. RcsC can sense stress directly and autophosphorylate, or it can be phosphorylated by the outer membrane sensor protein, RcsF. This makes the Rcs regulon responsive to stressors that affect both the inner and outer membrane.

Eleven genes within the *wca* (formerly *cps)* operon showed transient upregulation. The *wca* operon is responsible for the biosynthesis and excretion of colanic acid, the most common exopolysaccharide (EPS) produced by *Escherichia coli* and other *Enterobacteriaceae* (Grant 1969). Colanic acid has been implicated in survival outside the host and resistance to stressors including desiccation and chemical sanitizers (Ophir 1994; Samrakandi 1997). It has been suggested that stress from osmotic shock precedes

desiccation and that RcsC responds to this preliminary signal by activating the *wca* operon in preparation for desiccation (Sledeski 1996). The protective layer provided by

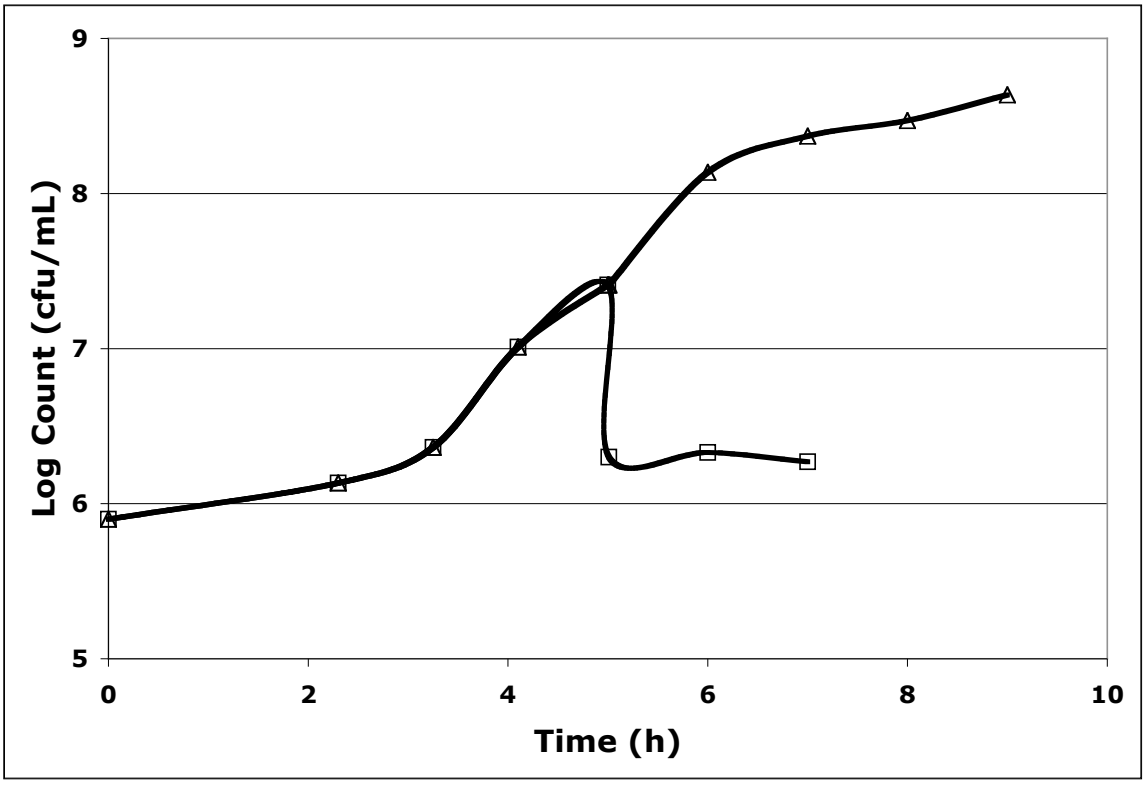

Figure 2. Growth of *Salmonella* Typhimurium LT2 in 5% TSB at 32ºC. Open squares represent the untreated (control) culture and open triangles represent the culture treated with pulsed electric filed (PEF) at 25.6kV/cm, 91us, 32ºC±2ºC. After PEF treatment, cells were incubated at 32ºC and aliquots were plated at the selected time points.

colanic acid may then prevent desiccation by binding water and creating a protective aqueous layer around the cell. Colanic acid production has also been shown to be induced by mutations that affect lipopolysaccharide synthesis and consequently result in alterations to the structure of the outer membrane (Parker 1992). The common factor among these inducers is damage to cell envelope components.

Table 1. Fold changes of genes involved in exopolysaccharide production and proton motive force maintenance showing transient upregulation in response to PEF treatment.

Gene symbol	Gene function	Fold change 3 min	30 min	60 min
***wca* operon**[1]				
wcaE	putative glycosyl transferase	1.2	5.1	1.9
wcaF	colanic acid acetyltransferase	1.2	8.0	2.3
wcaG	GDP-fucose synthetase	1.0	5.2	2.5
wcaH	GDP-mannose mannosyl hydrolase	1.1	5.9	2.6
wcaI	putative glycosyl transferase	1.0	5.5	1.9
wcaJ	UDP-glucose lipid carrier transferase	1.0	5.6	2.2
wcaJ	putative pyruvyl transferase	1.1	2.8	1.8
wcaK	putative glycosyl transferase	1.1	4.1	2.3
wcaL	colanic acid biosynthesis protein	1.1	2.0	1.4
***yjb* operon**[1]				
yjbE	outer membrane protein	1.3	4.6	4.9
yjbF	outer membrane lipoprotein	1.1	4.0	1.5
yjbG	periplasmic protein	1.1	3.3	1.6
yjbJ	stress-response protein	1.2	1.7	1.1
***psp* operon**				
pspA	phage shock protein PspA	1.1	6.5	4.0
pspB	phage shock protein B	1.1	6.7	3.7
pspC	DNA-binding transcriptional activator PspC	1.0	9.3	5.8
pspD	peripheral inner membrane phage-shock protein	1.1	7.2	3.2
pspE	thiosulfate:cyanide sulfurtransferase	1.1	8.0	3.0
pspG	phage shock protein G	1.2	3.1	2.3

[1]operon is under control of the Rcs regulon

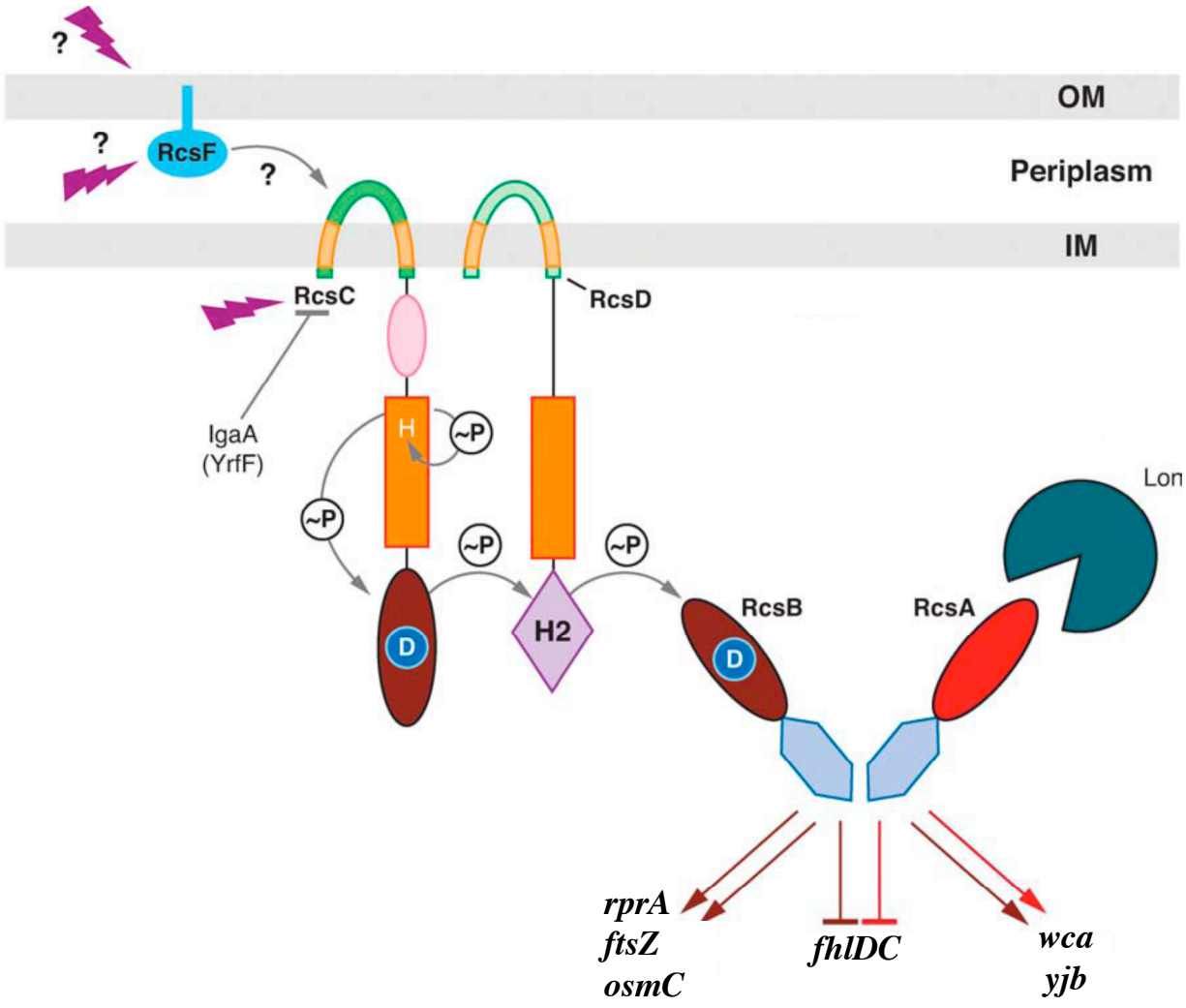

Figure 3. Functional components of the Rcs system. Signals may be transduced through the outer membrane protein RcsF or directly detected by RcsC. RcsC begins the phosphorlyation chain that eventually activates the transcription activator RcsB. RcsA may be required for binding of RcsB to certain promoter regions. Note positive regulation of *wca*, involved in colanic acid production and the negative regulation *flhDC*, the master flagella regulon.

The *yjb* operon, which is necessary for the production of an uncharacterized EPS also showed a transient increase in transcription. Sequence analysis of the *yjb* operon has shown the presence of genes involved in the production of substrates for EPS synthesis as well as potential β-barrel transporters for the export of EPS (Ferreires 2007: Zhai 2003).

However, none of the genes in the *yjb* operon show homology to the enzymes required for the biosynthesis of EPS. It is interesting to note that the genes of the *wca* operon responsible for the export of colanic acid were not upregulated. Although there have been no studies showing interaction between the protein components of the Wca and Yjb EPS systems, it would be interesting to examine the affinity of both the *wca* enzymes for *yjb* EPS substrates and the *yjb* membrane transporters for colanic acid, especially in light of their co-regulation under the Rcs regulon.

Proton motive force

The *psp* operon is responsible for the maintenance of proton motive force (PMF) under stress (Kleerebezem 1996). Proton motive force is made of both a pH component, that is dependent upon the concentration gradient of H^+ ions across the membrane, as well as an electrical potential component. Current understanding of the Psp system points to induction via alteration of the pH component of PMF. Therefore, upregualtion of the *psp* operon suggests the leakage of H^+ ions from the periplasm following PEF treatment. The proteins in the Psp system are arranged in a similar fashion to those of the Rcs system, with two membrane bound regulators, PspB and PspC that affect the activity of a transcriptional regulator, PspA. Under normal conditions, PspA acts as a transcriptional repressor of the *psp* operon (Weiner 1991). However, under stress conditions that cause a decrease in PMF across the plasma membrane, such as ethanol, and hyperosmotic shock, PspA is modified and repression of *pspABCDEF* is released (Brissette 1990). Mutations causing exported proteins to become trapped and holding the transport channels in an open position, leading to ion leakage across the inner membrane have also been shown to increase production of Psp proteins (Brissette 1990). *psp*

upregulation observed in this study may be caused by PEF induced denaturation of proteins within protein transport channels at the time of treatment, thus interrupting transport and preventing transport channels from closing. Previous studies have suggested that PspB is primarily responsible for activation during malfunction of protein transport, while PspC is more important during response to ethanol and osmotic shock (Weiner 1991). Investigation of the response of *pspB* and *pspC* strains to PEF may aid in further characterization of PEF induced membrane damage.

Potential cellular targets for combination treatment

Colanic acid (Meredith 2007) and likely the *yjb* EPS contain slight, yet numerous variations even within a single culture, potentially leading to difficulty in developing treatments that target them specifically. An alternative approach may be to develop compounds that target the excretion apparatus. Potential mechanisms to consider are binding of the interior of the excretion channels, causing them to remain open thus destroying the electrochemical gradient of already damaged cells. Such compounds could be added to a food system before PEF treatment or shortly after, preceding the upregulation of the *wca* and *yjb* export systems.

The PspA protein may also present a target for combination treatments. It has been shown that PspA once modified, forms a large oligomer that is directly responsible for the stabilization of PMF by stopping proton leakage into the cytoplasm (Kobayashi 2007). Development of treatments that prevent the oligerimization of PspA or those that prevent the activation of PspA may prevent recovery of cells.

PEF induced SOS response

Genes involved in the DNA damage induced SOS response showed a sustained increase in transcription beginning at the 30 min. time point. The apparent delay and/or persistence in the upregulation of these genes may be related to cellular detection of DNA damage. Cells detect DNA damage during replication of the chromosome. This process involves stalling of the replication machinery, which generates the DNA damage signal; single stranded DNA (Walker 2000). Therefore, the delay in the SOS response is directly related to the generation time of the culture being studied. The average generation time of *Salmonella* culture, within 4-6 hr of incubation, was approximately 30 min (Fig. 1). Assuming that the surviving cells maintained the same rate of individual growth, the SOS response would be expected to occur within 30 min, but probably no sooner than 10-15 min. post treatment. Strong and sustained upregulation of *sulA,* an SOS cell division inhibitor, also explains the cessation of growth observed in the surviving population of treated cells (Huisman 1980). From this study, it cannot be determined if PEF causes DNA damage directly, leaving the possibility that it is caused indirectly by an influx of damaging agents into the cytoplasm from either the periplasm or the growth medium.

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